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## ***HOX11L2/TLX3* is transcriptionally activated through T-cell regulatory elements downstream of *BCL11B* as a result of the t(5;14)(q35;q32)**

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### **Abstract**

The t(5;14)(q35;q32) chromosomal translocation is specifically observed in up to 20% of childhood T-cell acute lymphoblastic leukemia (T-ALL). It affects the *BCL11B/CTIP2* locus on chromosome 14 and the *RANBP17-TLX3/HOX11L2* region on chromosome 5. It leads to ectopic activation of *TLX3/HOX11L2*. To investigate the reasons of the association between t(5;14) and T-ALL, we isolated the translocation breakpoints in 8 t(5;14) patients. Sequence analyses did not involve recombinase activity in the genesis of the translocation. We used DNaseI hypersensitive experiments to locate transcriptional regulatory elements downstream of *BCL11B*. By transient transfection experiments, 2 of the 6 regions demonstrated *cis*-activation properties in T cells and were also effective on the *TLX3* promoter. Our data indicate that the basis of the specific association between t(5;14) and T-ALL lies on the juxtaposition of *TLX3* to long-range *cis*-activating regions active during T-cell differentiation.

### **Introduction**

Maturation of immunoglobulin (*Ig*) and T-cell receptor (*TCR*) genes is associated with somatic V(D)J recombinase rearrangements occurring during lymphoid development.<sup>1</sup> A protein complex containing both ubiquitously expressed and lymphoid-specific proteins (RAG1, RAG2, and TdT) catalyzes this process. The complex interacts with recombination sequences (RSs) that limit the segments to be rearranged. During the recombination process, the extremities are nibbled, and additional nucleotides (Ns) are added without template by the TdT.<sup>1</sup> During this process, which includes double-strand DNA break, the *Ig* and *TCR* genes are prone to illegitimate rearrangements and chromosomal translocations observed in human T-cell acute lymphoblastic leukemia (T-ALL) often involve the site of the *TCR* genes.<sup>2</sup> Molecular analyses of the chromosomal breakpoints of these translocations often showed

hallmark of recombinase activity and allowed the characterization of numerous T-ALL oncogenes.

Many of the oncogenic events specific for T-cell leukemogenesis result in the activation of transcription factors, such as *HOX11/TLX1*, *LMO2*, or *TAL1/SCL*.<sup>3,4</sup> A frequent event in T-ALL is the occurrence of a 90-kb deletion of chromosome 1 sequences, juxtaposing the first exon of the *SIL* gene, to the 5' part of the *TAL1* gene.<sup>5</sup> This rearrangement is observed in up to 20% of childhood T-ALL samples, and not in other ALL subtypes. The fusion points of the deletion bear the hallmarks of recombinase activity, which is therefore thought to be responsible for this rearrangement and provides the grounds for its specific association with T-ALL. Illegitimate recombinase activity is also responsible for at least some of the deletion affecting the *P16-ARF* tumor-suppressor locus observed in a T-ALL.<sup>6</sup>

Ectopic expression of the homeobox transcription factor *TLX3* was recently reported in childhood T-ALL, virtually always in association with genomic rearrangement of its locus.<sup>7-10</sup> It is mainly associated with a cryptic t(5;14)(q35;q32) chromosomal translocation that recombines the *RANBP17-TLX3* and *BCL11B* loci.<sup>11,12</sup> To uncover the mechanisms responsible for the translocation and ectopic expression of *TLX3*, and the reason for the specific association between t(5;14) and T-ALL, we analyzed the nucleotide sequence at the breakpoint of 8 examples of t(5;14). In addition, we identified *cis*-transcriptional regulatory elements lying downstream of *BCL11B*, which could be involved in the up-regulation of *TLX3* following the translocation.

## Materials and Methods

Samples were obtained with informed consent, in accordance with the Declaration of Helsinki. Approval for this study was given by the INSERM review board. The hematologic and immunophenotypic characteristics and conventional karyotypes of the 8 T-ALL patients with t(5;14)(q35;q32) are listed in Table S1, available on the *Blood* website (see the Supplemental Materials link at the top of the online article). The procedure for DNaseI experiments is described in Figure S2. DNA analyses were performed using standard techniques. DND41, HPB-ALL, and Jurkat are T-ALL-derived cell lines; DND41 and HPB-ALL bear variant t(5;14).<sup>12,13</sup> The primers used are listed in Table S2. Inverse polymerase chain reaction (PCR) was performed as described previously,<sup>12</sup> and classical PCR was then used to confirm breakpoint location and sequence. Sequence analyses were performed locally or at <http://genome.ucsc.edu> or <http://www.ncbi.nlm.nih.gov/gate1.inist.fr>. Appropriate fragments were cloned in pGL3 backbone (Promega, Madison, WI). Cells ( $2.5 \times 10^5$ ) were transfected with 0.5 to 2  $\mu$ g reporter gene constructs using Lipofectine (Invitrogen, Frederick, MD), and the luciferase activity was determined using luciferase assay system (Promega) after 24 hours. A representative experiment is shown, but every experiment was repeated at least 3 times in duplicate. The results were similar with or without normalization (using a thymidine kinase promoter-driven -galactosidase reporter).

## Results and Discussion

### Cloning of the chromosomal translocation breakpoints

Approximate mapping of the breakpoints was done by fluorescence in situ hybridization (FISH), analyses and more precise localization was achieved by Southern blotting analysis using single-copy probes (data not shown and [Figure 1A](#)). The translocation breakpoints were amplified from patient's genomic DNA using an inverse PCR approach and

analyzed by direct sequencing. Both translocation breakpoints (der5 and der14) could be analyzed in 7 cases and the fusion points exhibited complex features in only patient 1 (Figure S1). Most of the fusion points exhibited N nucleotides. In patients 3 and 7, no N nucleotides were observed, suggesting that the translocation could have occurred in a cell that did not express the TdT. Of importance, no convincing RS-like sequences were observed at the translocation breakpoints, suggesting that the recombinase activity is not involved in the occurrence of the translocation.

### **DNAse1 hypersensitive site (HSS) mapping**

It has been proposed that the transcriptional activation of *TLX3* could result from *cis*-activation of the gene by a *BCL11B* transcriptional regulatory element, juxtaposed to *TLX3* following the translocation.<sup>12,15</sup> Within the hematopoietic system, expression of the *BCL11B* gene is restricted to the T-cell differentiation pathway.<sup>7,16</sup> It encodes a transcription factor essential to thymocyte differentiation.<sup>17</sup> The location of the translocation breakpoints in patients 1 and 6 pinpointed to a 120-kb region of chromosome 14 susceptible to contain this putative minimal region. A previously described breakpoint<sup>15</sup> allowed us to narrow down this region to 58kb lying more than 920-kb downstream of *BCL11B* polyadenylation site. We used DNAse1 hypersensitive experiments to identify active regulatory elements in this region. As shown in [Figure 1B](#), 5 HSSs were observed in T-ALL cell lines (DND41 and Jurkat), but were absent or weaker in the non-T-cell lines Nalm6 and K562 (Figure S2).

To investigate for *cis*-activating properties, 1.5-kb fragments encompassing the HSSs were subcloned in pGL3-luciferase vector and tested for activation of the SV40 promoter. Only 2 regions (H3 and H4) showed enhancement of SV40 promoter activity in HPB-ALL ([Figure 2A](#)). Fine mapping of the HSS and luciferase reporter assays allowed us to map the active sequences within 464-bp (H3\*) and 482-bp (H4\*) fragments (data not shown and [Figure 2B](#)). As expected, *cis*-activation was observed in T-cell lines, but not in B-cell or myeloid cell lines. We next wanted to ensure the activity of these sequences on the *TLX3* promoter. A 504-bp fragment within the 5' side of *TLX3* was subcloned in promoter-less pGL3-luciferase vector (Figures S3 and S4). As shown in [Figure 2C](#), H3\* and H4\* sequences stimulated the *TLX3* promoter, which exhibited only a weak activity on its own in T-cell lines. Taken as a whole, our results identify T-lymphoid-restricted (within the hematopoietic system) *cis*-activating elements downstream of *BCL11B* that are juxtaposed to the *TLX3* locus as a result of the t(5;14).

Our observations suggest that the recombinase activity is not responsible for the occurrence of the t(5;14) in contrast with the *SIL-TAL1* deletion. They rather suggest that the reason for the association between t(5;14) and T-ALL is the juxtaposition of *cis*-transcriptional regulatory elements that are specifically active during T-cell differentiation. In keeping, murine *Bcl11b* is very highly expressed at starting from DN2 (and latter) stage of thymic development (data not shown).

The t(5;14) situation is the most frequent example observed in T-ALL that results in bona fide *cis*-activation due to enhancer juxtaposition. The frequent *SIL-TAL1* fusion leads to transcription of *TAL1* from the *SIL* promoter.<sup>5</sup> The breakpoints of chromosomal translocation

affecting the TCR locus frequently affect the 5' part of the oncogene on the partner chromosome, and result in the transcription from diversity region or cryptic promoters close to the translocation breakpoints.<sup>18-21</sup> We provide here only qualitative notions, and additional work will be necessary to fully characterize the *BCL11B* gene *cis*-regulating elements.<sup>22,23</sup>

However, it is tempting to speculate that the activity of these transcriptional regulatory regions represents a therapeutic target in t(5;14) patients.

## Authorship

Contribution: X.-Y.S., V.D.-V., I.A.-S., C.L., and I.R.-W. performed research and analyzed the data; P.B., M.L., M.L.-P., and F.M. provided vital reagents; R.B. and S.P.R. analyzed the data and reviewed the paper; O.A.B. and V.P.-L. designed research, analyzed data, and wrote the paper.

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## Footnotes

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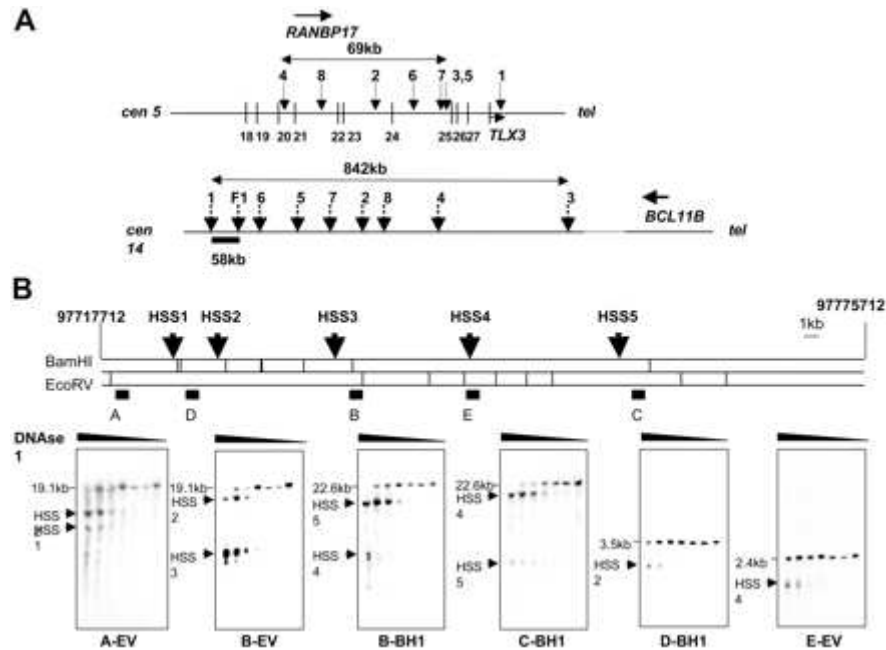
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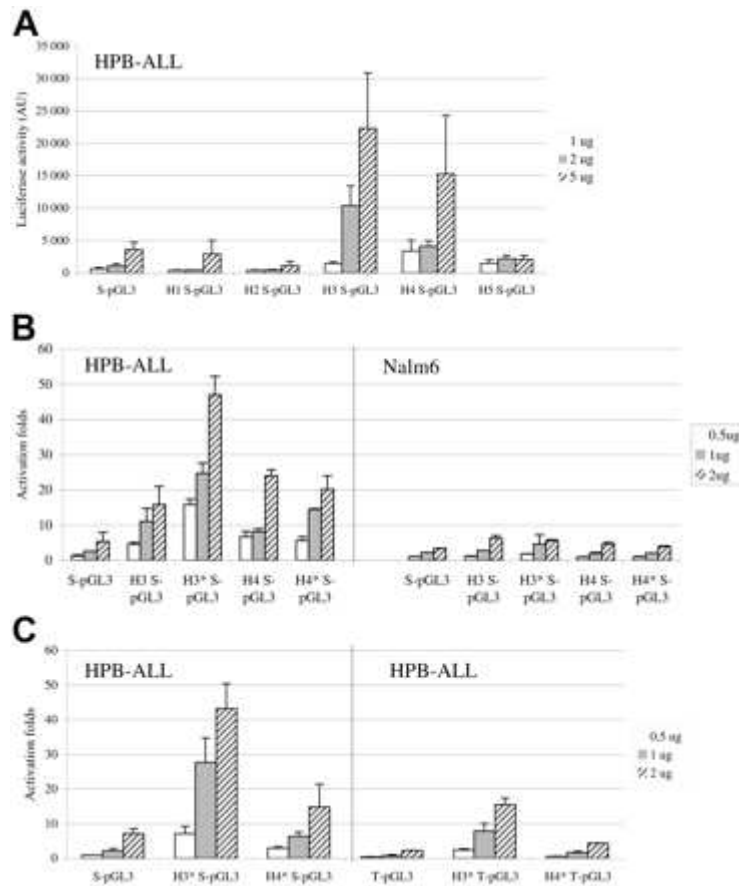
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**Figure 1.. Structural analyses of the loci involved in t(5;14) identify putative transcriptional cis-regulatory elements.** (A) Localization of the translocation breakpoints on chromosome 5 (top) and on chromosome 14 (bottom). Vertical arrows indicate the location of the breakpoints. The number refers to the patient's number. The majority of chromosome 5 breakpoints is located in a 69-kb area between exons 20 to 25 (shown by vertical lines) of *RANBP17*. A horizontal arrow indicates the sense of transcription of the genes. The chromosome 14 breakpoints are spread over 842 kb downstream of *BCL11B*, in a region devoid of any obvious gene.<sup>7,14</sup> The nucleotide sequences of the breakpoints are presented in Figure S1. Note that the breakpoint of patient 1 is located in the 3' untranslated region of *TLX3*. The chromosome 14 region that is juxtaposed in *cis* to the *TLX3* gene in every t(5;14) patient characterized here is located between the breakpoints of patients 1 and 6. F1 indicates the breakpoint of a complex t(7;14;15) reported previously<sup>15</sup> that allows the breakpoint to narrow the "critical" region to a 58-kb area indicated by a black rectangle. (B) DNase1 mapping experiments in the chromosome-14 region predicted to contain transcriptional regulatory elements. (Top) Map of the 58-kb chromosome-14 area analyzed in this experiment, showing *EcoRV* (EV) and *BamHI* (BHI) sites and the probes used. The numbers refer to chromosome 14 genomic sequences (NCBI Build36; NCBI, Bethesda, MD). (Bottom) Southern blot analysis of DNase1 hypersensitivity experiments using DND41 cells. Increasing concentrations of DNase1 reveal 5 hypersensitive sites (the corresponding fragments are shown by arrows). The sizes of the germ-line fragments are indicated. Additional experiments are presented in Figure S2.





**Figure 2.. Identification of cis-regulator elements active on the TLX promoter active in T-cell lines.** (A) Transcriptional regulatory activity of HSSs 1 to 5. Fragments encompassing the HSSs were subcloned in pGL3 (S-pGL3: containing an SV40 promoter) and tested by transient transfection in HPB-ALL. Luciferase activities are in arbitrary units (AU) and not normalized. (B) Fragments from H3 and H4 were tested for transcriptional activity using S-pGL3 luciferase reporter vector in HPB-ALL (T-ALL; left panel) and Nalm6 (BCP-ALL; right panel) cell lines. Three different concentrations of vector were used. Values are indicated as fold of activation with respect to 0.5  $\mu$ g S-pGL3 vector independently in each cell

line. Data were normalized using a  $\beta$ -galactosidase reporter. (C) H3\* and H4\* are active on SV40 (left panel) and *TLX3* (right panel) promoters. Normalized values are indicated as fold of activation with respect to 0.5  $\mu$ g S-pGL3 vector (left panel) or 0.5  $\mu$ g T-pGL3 (right panel). The nucleotide sequence of fragments H3\* and H4\* is shown in Figure S5. Error bars indicate standard deviations.

